

The Diagnostic Value of Cytokeratins and Carcinoembryonic Antigen Immunostaining in Differentiating Hepatocellular Carcinomas from Intrahepatic Cholangiocarcinomas

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Abstract

Aim. To study the differences between the hepatocellular carcinoma (HCC) and peripheral type of cholangiocarcinoma (CHC) using cytokeratin (CK) and carcinoembryonic antigen (CEA) expressions and to assess their accuracy on paraffin sections in the differential diagnosis. **Material and method.** The following antibodies were analyzed: AB1 complex (anti CK9-CK20), AB2 complex (anti CK1-CK8), pCEA, and the monoclonal antibodies against cytokeratins CK7, CK8/18, CK17 and CK19. In the immunohistochemical studies, 15 selected surgically resected liver tumors, 10 HCCs and 5 CHCs, with well established diagnosis (by morphological criteria) were included. Other markers, such as AFP si CA 19-9, were not available. **Results.** No CHC, but 50% of HCCs were positive for CEA, presenting a canalicular staining pattern. For CK 7, all but one (which was focally positive), meaning 80% of CHCs were diffusely positive, whereas only two HCCs were positive. For CK 19, 80% of CHCs were diffusely positive, while all but two HCCs (a moderately and a poorly differentiated tumor) were negative. For CK 8/18, 70% of HCCs were diffusely positive, but only 20% of CHCs were positive. For CK 17, 60% of CHCs were positive, while all HCCs were negative. 80% of CHCs were positive for AB1 anti-CKs complex, whereas only 50% of HCCs were positive, and relating to AB2 anti-CKs complex, 50% of HCCs were diffusely positive and only 20% of CHCs. **Conclusion.** The immunohistochemical expression of CKs and CEA might be considered helpful in addition to other diagnostic criteria for the differential diagnosis of primary carcinomas of the liver, especially in difficult cases.

Key words

Cholangiocarcinoma - hepatocellular carcinoma - monoclonal antibody - citokeratin - carcinoembryonic antigen - immunoperoxidase stains

Rezumat

Scopul studiului. Stabilirea diferențelor dintre hepatocarcinom (HCC) și colangiocarcinomul (CHC) periferic în ceea ce privește expresia unui grup de markeri imunohistochimici (citokeratine și antigen carcinoembrionar) în vederea stabilirii valorii lor diagnostice. **Material și metodă.** Au fost utilizate următoarele tipuri de anticorpi: complexul AB1 (anticitokeratine 9-20), complexul AB2 (anticitokeratine 1-8), CEA policlonal, anti CK 7, CK8/18, CK17, CK19. În acest studiu au fost incluși 15 pacienți rezecați, respectiv 10 cu HCC și 5 cu CHC, al căror diagnostic clinic și morfopatologic era bine cunoscut. Alți markeri utili ca alfa-fetoproteina și CA 19-9 nu au fost disponibili. **Rezultate.** 50% dintre esantioanele de HCC au fost pozitive pentru p CEA (pattern de colorare de tip canalicular), în timp ce toate mostrele de CHC au fost negative pentru acest marker. Pentru combinația CK8/18, 70% din HCC au fost difuz pozitive, în timp ce CHC au fost pozitive doar într-un caz (20%). Cu excepția unui singur caz (pozitiv focal), toate esantioanele de CHC, reprezentând 80% din cazuri, au fost pozitive pentru CK7, în timp ce HCC au fost negative, cu excepția a două cazuri pozitive focal. În cazul CK19, CHC a fost pozitiv difuz în timp ce HCC au fost negative, cu excepția a două cazuri (tumori moderat sau slab diferențiate). Pentru CK17, 60% din cazurile de CHC au fost pozitive în timp ce toate cazurile de HCC au fost negative. 80% din cazurile de CHC și 50% din HCC au fost pozitive pentru complexul AB1, iar pentru complexul AB2 20% din cazurile de CHC și 50% din cazurile de HCC au fost pozitive. **Concluzii.** Imunohistochimia pentru CK 8/18, CK7, CK 17, CK 19 și CEA este utilă pentru diagnosticul diferențial dintre HCC și CHC - în special în cazuri greu de diagnosticat morfopatologic.

Introduction

The aim of immunohistochemistry is to identify an antigen by a specific antibody labeled with fluorescent material or enzyme which is detected using a chromogen

(1). This is an important tool in histopathology, not only for the recognition of infectious agents, but also for the identification of cell lineages within a specific organ, or in typing malignant tumors, and has an unlimited role in research (2). There is probably no other method that revolutionized the field of pathology in the past 50 years. Different immunohistochemical techniques are available. Most of them can be applied on fine needle aspirates, fresh frozen tissue and paraffin embedded material. Antigen retrieval methods such as trypsinization and microwave heating are almost routinely used for better results (3). It is important to use controls or to look for intrinsic controls for foolproof evaluation. Indirect immunoperoxidase, three-step peroxidase, anti-peroxidase (PAP) and avidin-biotin complex are the available techniques.

Cytokeratins are a highly complex subclass of the intermediate filaments gene family and comprise more than 20 different polypeptides. They are largely classifiable as type A or class I (CK9-CK20) and type B or class II (CK1-CK8) keratin genes (2,4,5). In the individual cell, there are 2 to 10 different CKs. In the normal human liver, CK profile shows a characteristic distribution: CK 8 and 18 are present in hepatocytes, whereas CK 7, 8, 17, 18 and 19 are present in bile duct cells. Therefore, CK 7 and 19 are useful markers of bile ducts (2,4). CK profiles of the human liver have been widely related to the study of development of the biliary tree and the pathologic study of various hepatobiliary diseases (4,6-8).

Primary epithelial malignancies require an important differential diagnosis with other tumors of the liver and often cause considerable diagnostic dilemmas, thus prompting the use of immunohistochemical stains. In the last decade, various researchers (9,10) have recommended a panel of immunohistochemical markers to differentiate hepatocellular carcinoma (HCC) from cholangiocarcinoma (CHC). The cytokeratin (CK) profile is helpful in this respect. Although CKs 7 and 19 are not expressed in normal hepatocytes, being specific for CHC (9,11,12), focal expression can be seen in HCCs, and they can be abundant in the fibrolamellar type of HCC (9,13). Cytokeratins 7, 19 and 20 are not present in most HCCs, but they exist in many adenocarcinomas, including CHC. The results of the immunohistochemical staining for CKs 7 and 20 offer a first suggestion for the primary origin of the tumor (12,14), i.e. a CK 7+/CK 20- profile indicates a peripheral type of CHC, while a CK 7-/CK 20- profile corresponds to HCC. Cytokeratins 8 and 18 are immunoreactive in HCC, but the staining, which is submembranous, can be very weak. In one study it has been shown that the monoclonal antibody (AB1) to all acidic CKs (type I) decorates normal bile duct epithelium and CHCs, but does not stain normal and neoplastic hepatocytes (12,15). Thus, a panel of CKs may be required for accurate assessment.

Polyclonal CEA produces a specific pattern of canalicular staining in HCC, but this may be combined with non-canalicular membrane and cytoplasmic staining which are not specific for HCC (16). The diagnosis of HCC is suggested

by the type of tumor cells: resembling hepatocytes, producing bile, having a trabecular growth or a sinusoidal vascularisation pattern. But pseudoglandular formation, clear cell change and poor differentiation could make this difficult (17). The sclerosing variant of HCC can mimic CHC. Also, abundant formation of acinar structures (rosettes) can cause difficulties in differentiating HCC from CHC. Detection of canalicular differentiation is much better, because even poorly differentiated tumors show this trait, often in the form of intracellular aberrant canaliculi which are seen as intracellular dots on pCEA stain (14). Better differentiated tumors show nicely structured canaliculi, as pCEA-positive lines in between adjacent tumor cells or in the tumoral trabeculae. In contrast, poorly differentiated CHCs show a diffuse cytoplasmic staining pattern. For the interpretation of the canalicular stainings, it is helpful that surrounding non-tumoral parenchyma is present as an internal control (4).

Material and methods

Patients and specimens

We selected from the files of the Department of Pathology, Clinical Institute Fundeni, the resected specimens of primitive epithelial malignant hepatic tumours and these were reviewed retrospectively to evaluate the differences of the CKs and CEA expressions between HCCs and CHCs. We also investigated the usefulness of their expression in developing the differential diagnosis between them.

Fifteen specimens of those with sufficient paraffin-embedded tissue for immunohistochemical studies were collected. The tissue sections and medical records of these cases were reviewed to ensure that the morphology and clinical information were consistent with primary carcinomas of the liver. The results were 10 cases of HCC and 5 cases of peripheral CHCs. The HCCs were classified according to Edmonson-Steiner's grading system (18), while CHCs were classified in ordinary three-stage grading system. The personal data of the patients, the pathologic features, the largest dimension of the tumor and staining results with the antibody panel are shown in Table I.

The specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Several 5- μ m thick step serial sections were obtained from each paraffin block and were stained one with hematoxylin-eosin, and the remainder were subjected to the immunohistochemical staining for CKs and CEA.

Immunohistochemistry

Immunohistochemical studies were performed using primary antibodies from Neo Markers – Labvision and secondary biotinylated antibodies and detection kit were from Neo Markers when we used DAB (3,3'-diaminobenzidine) and from Biomedica (AutoProbe II Universal Kit) when we used AEC (3-amino-9-ethylcarbazole).

Quantification of immunohistochemistry

Immunostaining was considered positive when more than 5% of cells were stained. When it was detected, the CK

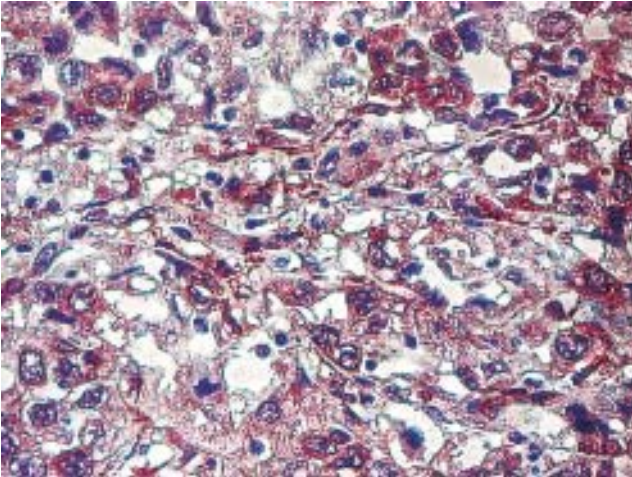


Fig.1 HCC poorly differentiated, CK 8/18, AEC chromogen, magnification 400X.

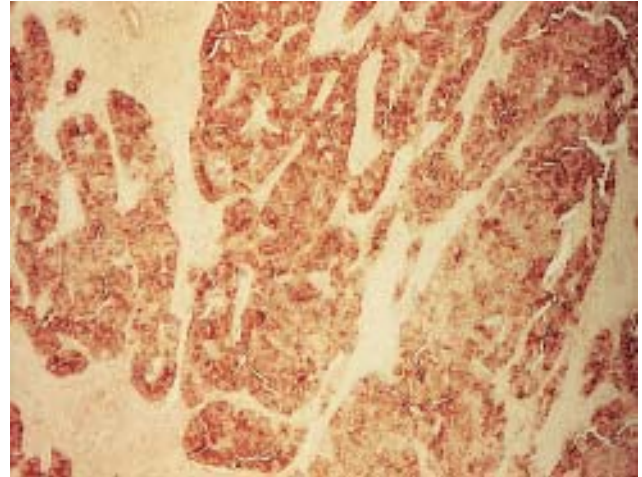


Fig.4 CHC well differentiated, CK 17, DAB chromogen, magnification 100X.

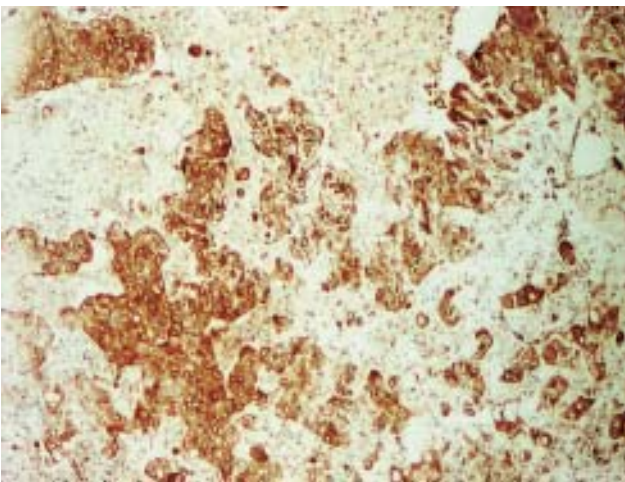


Fig.2 HCC poorly differentiated, CK 8/18, DAB chromogen, magnification 100X.

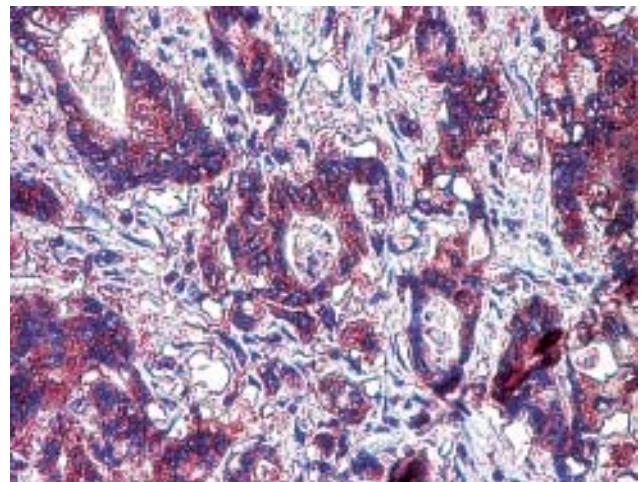


Fig.5 CHC well differentiated, CK 19, AEC chromogen, magnification 200X.

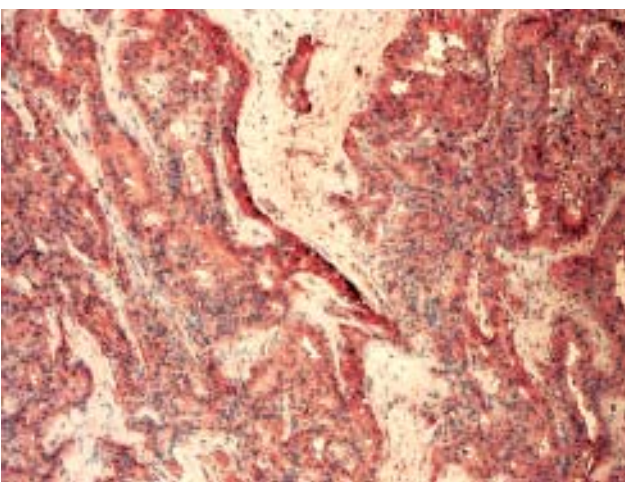


Fig.3 CHC well differentiated, CK 7, AEC chromogen, magnification 200X.

expression was semi quantitatively classified into 3 scores according with the percentage of positive cells: - (negative), ± (focally positive) and + (diffusely positive).

Results

Non-neoplastic area of the liver

The antiserum to CEA stained the luminal membrane of the bile ducts, the bile ductules and the canaliculi. The hepatocytes were positive for CK 8 and 18. Intrahepatic bile ducts as well as peribile duct glands, including proliferating glands, were positive for CK 7, 8/18, 17 and 19.

Hepatocellular carcinoma

In 50 % of HCCs, a bile canalicular staining pattern was observed with the antibody to CEA that was similar to normal liver but it was sometimes associated with cytoplasmic staining. The intensity of staining was correlated with the grade of differentiation: grade I-II – deeply positive, while grade III-IV was generally poorly positive. Neoplastic hepatocytes presented with two exceptions – moderately, respectively, poorly differentiated HCC – a negative reaction with the antibody to CK 19. This staining pattern was elicited throughout the tumors, including areas where the diagnosis would not have been clear-cut if these areas were the only ones available for study, e.g. following a needle biopsy.

Three of our cases displayed a pattern of pseudoglands, which were negative for CK 19 and exhibited a paraluminal membrane staining to the antiserum to CEA. One tumour displayed focal clear-cell areas and other had a prominent scirrhous stroma. Both were negative for CK 19. Fifty percent of HCCs were also positive for AB1 complex of CKs, whereas 80% of CHCs were positive. A small percentage (33%) of HCCs was positive for AB2 anti-CK antibody, whereas 60% of CHCs were positive. Seventy percent of HCCs was positively stained with anti-CK 8/18 monoclonal antibody (Figs. 1, 2). All HCCs were negative at the staining with anti-CK 7 and anti-CK 17 monoclonal antibodies.

Cholangiocarcinoma

All CHCs displayed a weak and predominantly paraluminal staining of the cytoplasmic membrane with the anti-CEA serum, but no biliary canalicular pattern was noted. Only 20 % of CHCs (one poorly differentiated case) were positive for anti CK 8/18 antibody in contrast to 70% of HCCs. All CHCs were positive for CK 7 (Fig.3), most of them

showing a diffuse pattern of positive staining, only one moderately differentiated tumour had focal positive staining.

There was no difference in CK 7 expression referring to the grade of differentiation of the tumour. The single case of squamous cell carcinoma was diffusely positive for CK 7, too.

Four cases (80%) of CHCs showed focal or diffuse intracytoplasmatic expression for CK 19 (Fig.5).

The amount of CK 19 seems to be correlated with the grade of differentiation of the tumour, being highest in well-differentiated CHCs, relatively low (focal) in moderately differentiated and absent in poorly differentiated cases. Similar staining behaviour was seen regarding CK 17, 60% of CHCs demonstrating positivity (diffuse) excepting one – the squamous case – that was focally positive (Fig.4). Moreover, positive staining with CK 17 was detected only in well differentiated CHCs. 80% of CHCs were positive stained with AB1 anti-CKs antibody, but only 20% of CHCs were positive in case of AB2 anti-CKs antibody staining.

Table I Results

No	Age (y)	Sex	Pathologic diagnosis	Tumour size(cm)	CK-AB1	CK-AB2	CK 8/18	CK 7	CK 17	CK 19	CEA (Be)
1	60	M	Squamous CHC – G1	2	+	+	-	+	±	+	-
2	69	F	CHC – G1	12	+	-	-	+	+	+	-
3	45	M	CHC – G1	18	±	+	-	+	+	+	-
4	72	F	CHC – G3	3	-	+	+	+	-	-	-
5	61	F	CHC – G2	4	+	-	-	±	-	±	-
6	56	M	HCC – E/S II-III	4	+	-	+	-	-	-	-
7	75	F	HCC – E/S II-III	5	-	+	±	-	-	-	-
8	51	M	HCC – E/S II-III*	5	±	-	-	-	-	-	-
9	18	M	Fib HCC – E/S II-III	16	±	-	±	±	-	±	+
10	50	M	HCC – E/S III-IV	20	-	-	-	+	-	±	±
11	65	M	HCC – E/S III	3	-	+	±	-	-	-	±
12	68	F	HCC – E/S II-III**	9	-	-	±	-	-	-	-
13	18	M	Fib HCC – E/S II-III	17	+	-	+	-	-	-	+
14	48	F	HCC – E/S I-II	7	+	+	+	-	-	-	+
15	52	M	HCC – E/S II-III	10	+	-	-	-	-	-	-

Discussion

There have been many reports on CKs expression in portal tracts and fibrous septae in various hepato-biliary diseases (11,19) and in hepatic neoplasms (20). Biochemical analysis has revealed that human hepatocytes contain only two CKs – CK8 and 18, as identified in the catalog of Moll (4). These two CKs were demonstrated in HCC by two-dimensional gel electrophoresis. The monoclonal antikeratin antibody AB1 recognizes all acidic CKs at 1 to 8 (subfamily A or type I), whereas AB2 antikeratin antibody recognizes all basic CKs (subfamily B or type II), but it usually does not react with HCCs (14) or hepatocytes (21). CK 19 is characteristically detected in biliary cells, but not in hepatocytes (5,22). There have been several reports about the immunohistochemical expression of CKs and CEA in primary liver carcinoma, many of them related to the usefulness of this markers in differentiating HCC from CHC (5,7,9,23). Indeed, neoplastic cells usually retain the CK pattern of their nontumoral counterparts, and CHC and HCC cells are known to confirm this rule (5,9,17,21,23). Monoclonal antibodies against several cytokeratins have been reported to stain only 10% of HCCs, but up to 80% of CHCs (24,25). A recent investigation on primary epithelial malignant tumors of the liver has shown that CHCs were stained positively for CK 8, 18 and 19, while HCCs were positive for CK 18 and negative for CK 7 and 19 (24-26).

These immunohistochemical investigations can be related to biochemical studies that have established that normal and neoplastic hepatocytes enclose a unique subset of CKs composed of subtypes 8 and 18 (9,26). On the other hand, CK 19 is widely expressed in most CHCs and epidermoid carcinomas (11,12). In this study, it was found that CK 7, 8/18, 17 and 19 were expressed diffusely in non-neoplastic intrahepatic biliary tree including normal and proliferated peribiliary glands, while CEA was expressed in biliary ducts and ductules. In 80% of CHC cases examined, all of CK 7, 8/18 and 19 were expressed in neoplastic cells. Among these, CK 7, 8 and 19 were detected variably in most of the cases of CHC examined, suggesting that neoplastic cells retained these CK. By contrast, 20% of CHCs exhibited CK 18 expression, suggesting that the expression of it was lost in the remaining cases of CHC, after neoplastic transformation. Diffusely expression of CK 19 was correlated with histological differentiation of the CHC. The unique case of squamous cell type of diffuse CHC revealed CK 7 and 19, while the CK 17 expression was lower. In this study it was found that the CKs profile of HCC was different in incidence and distribution as compared with CHCs. In HCCs, positive aberrant CK7 (20%) was associated with CK19-coexpressing pseudoglandular pattern suggesting ductular metaplasia. This focal reactivity of some neoplastic hepatocytes for CK 19 should support the hypothesis that mixed hepatocellular and cholangiocellular tumors as well as some CHCs, are more or less metaplastic HCCs. Similar observations were already reported by van Eyken (5).

In our study, however, 50% of HCCs and 80% of CHCs

were positive with AB1. AB2 stained 30% of HCCs and 60% of CHCs. According to Battifora, AB2 works well in alcohol-fixed tissues but is insensitive in formalin-fixed specimens (27). Battifora has found that 70% of HCCs were positive with AB1. The use of monoclonal antibodies against the two groups of CKs (AB1 and AB2) resulted in nonreliable data.

Many studies have indicated the utility of CEA in distinguish HCC from CHC or metastatic carcinomas (9,16,17,20,25,26). In our study we have confirmed previous reports showing the specificity of the canalicular staining pattern for CEA in HCC. This pattern was elicited in 50% of the cases that have been studied and was never observed in CHCs. Many HCCs with positive cytoplasmic staining by pCEA revealed intense biliary canalicular immunoreactivity. Thus, at least part of the cytoplasmic staining may be a secondary phenomenon. Results of this study indicate and support the assertion in the literature that the biliary canalicular staining pattern is useful in confirming a diagnosis of HCC. The rate and type of pCEA positivity in this study are comparable to that reported in the literature (9,16,17) which ranged from 40 to 90% (17). Some authors have indicated that poorly differentiated HCCs tend to be negative for pCEA, and therefore, this marker may not be useful in confirming the diagnosis of HCC (16). However, in our study, 70% of moderately differentiated and all poorly differentiated HCCs, exhibited pCEA. A negative pCEA stain does not exclude HCC – 50% of our HCCs cases were negative.

Conclusions

The present study suggests that the profile of CK7 and CK 19-positive biliary cells is different in CHCs and HCCs. Moreover, the panel of CKs and CEA (with biliary canalicular pattern of staining) was helpful in differentiating CHC from HCC. Biliary epithelial cells retain CK 7, 17 and 19 after neoplastic transformation in almost all cases, while CK 8/18 may be useful in HCC cases. The combined analysis of CK 7, 8, 18, 17, 19 and CEA was useful in the differential diagnosis of HCC from CHC. The AB1 and AB2 monoclonal antibodies have demonstrated a lower sensibility than expected and together with a lack of specificity do not make these markers useful for differentiation of primary epithelial malignancy of the liver.

The immunohistochemical expression of CKs and CEA is useful, in addition to other diagnostic criteria, for establishing a differential diagnosis in the case of primary and metastatic carcinomas of the liver.

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